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## **The TvPirin gene is necessary for haustorium development in the parasitic plant *Triphysaria versicolor***

Bandaranayake, P C G ; Tomilov, A ; Tomilova, N B ; Ngo, Q A ; Wickett, N ; DePamphilis, C W ; Yoder, J I

**Abstract:** The rhizosphere is teemed with organisms that coordinate their symbioses with plant roots through chemical signals. Chemicals also mediate associations between roots of different plants; the most obvious being those between parasitic Orobanchaceae and their plant hosts. These parasitic plants use specific molecules provided by host roots to initiate the development of haustoria, invasive structures critical for plant parasitism. We have taken a transcriptomics approach to identify parasitic plant genes associated with haustorium signaling. We previously identified a gene, TvPirin, which is transcriptionally up-regulated in roots of the hemiparasite *Triphysaria versicolor* that had been exposed to the haustorium inducing molecule 2, 6-dimethoxybenzoquinone (DMBQ). Because TvPirin shares homology with proteins associated with environmental signaling in other plants, we hypothesized that TvPirin may function in host factor recognition in parasitic plants. We tested the function of TvPirin using hairpin mediated RNA interference and found that reducing TvPirin transcripts in *T. versicolor* roots resulted in significantly less haustoria development. We then determined the steady state transcript levels of other root expressed genes and found that several were reduced in TvPirin silenced roots in the absence of inducer. However the up regulation of genes by DMBQ was similarly regulated in control and TvPirin silenced roots. Phylogenetic investigations showed that TvPirin homologs are present in most flowering plants and we found no evidence of parasite specific gene duplication or expansion. We propose that TvPirin is a generalized transcription factor associated with the expression of several genes, some of which are involved in haustorium development.

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# The *TvPirin* Gene Is Necessary for Haustorium Development in the Parasitic Plant *Triphysaria versicolor*<sup>1[C][W][OA]</sup>

Pradeepa C.G. Bandaranayake, Alexey Tomilov, Natalya B. Tomilova, Quy A. Ngo, Norman Wickett, Claude W. dePamphilis, and John I. Yoder\*

Department of Crop Science Faculty of Agriculture, University of Peradeniya, Sri Lanka 20400 (P.C.G.B.); Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, California 95616 (A.T.); Department of Plant Sciences (N.B.T., J.I.Y.) and Department of Molecular Biosciences, School of Veterinary Medicine (A.T.), University of California, Davis, California 96516; Institute of Plant Biology, University of Zurich, Zurich CH-8008, Switzerland (Q.A.N.); Department of Biology and Huck Institutes of Life Sciences, Penn State University, University Park, Pennsylvania 16802 (N.W., C.W.d.); and Plant Science Center, Chicago Botanic Garden, Glencoe, Illinois 60022 (N.W.)

The rhizosphere is teemed with organisms that coordinate their symbioses using chemical signals traversing between the host root and symbionts. Chemical signals also mediate interactions between roots of different plants, perhaps the most obvious being those between parasitic Orobanchaceae and their plant hosts. Parasitic plants use specific molecules provided by host roots to initiate the development of haustoria, invasive structures critical for plant parasitism. We took a transcriptomics approach to identify parasitic plant genes associated with host factor recognition and haustorium signaling and previously identified a gene, *TvPirin*, which is transcriptionally up-regulated in roots of the parasitic plant *Triphysaria versicolor* after being exposed to the haustorium-inducing molecule 2,6-dimethoxybenzoquinone (DMBQ). Because *TvPirin* shares homology with proteins associated with environmental signaling in some plants, we hypothesized that *TvPirin* may function in host factor recognition in parasitic plants. We tested the function of *TvPirin* in *T. versicolor* roots using hairpin-mediated RNA interference. Reducing *TvPirin* transcripts in *T. versicolor* roots resulted in significantly less haustoria development in response to DMBQ exposure. We determined the transcript levels of other root expressed transcripts and found that several had reduced basal levels of gene expression but were similarly regulated by quinone exposure. Phylogenetic investigations showed that *TvPirin* homologs are present in most flowering plants, and we found no evidence of parasite-specific gene duplication or expansion. We propose that *TvPirin* is a generalized transcription factor associated with the expression of a number of genes, some of which are involved in haustorium development.

The Orobanchaceae comprises roughly 90 genera of facultative and obligate parasitic plants that directly invade host roots to rob them of water and nutrients (Kuijt, 1969; Musselman, 1980; Nickrent, 2011). The Orobanchaceae invade their hosts via haustoria, par-

asitic organs that attach to and penetrate host tissues. Haustoria later develop physiological connections between host and parasite vasculature systems that provide the conduit for the transfer of metabolites, proteins, nucleic acids, and viruses between host and parasite (Westwood et al., 2009). Root parasitism can be debilitating to host plants, and some of the world's most devastating agricultural pests are weedy Orobanchaceae (Parker and Riches, 1993).

Hauستoria develop on parasite roots in response to contact with host roots or host recognition molecules termed xenognosins (Lynn et al., 1981). Haustorium ontogeny can be observed under a microscope by exposing aseptically parasite roots in vitro to host exudates or purified xenognosins (William, 1961; Atsatt et al., 1978; Riopel and Timko, 1995). Within minutes of xenognosin exposure, the parasite root stops elongating and its growth is redirected toward a radial expansion of the root tip. After a few hours, the root tips noticeably swell and epidermal hairs begin to elongate over the swollen root tissue. Depending on the species, the swelling and hair proliferation are localized at the

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\* Corresponding author; e-mail jiyoder@ucdavis.edu.

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apex of the emerging radical or at lateral positions just behind the root cap. In the presence of a host, the haustorial hairs attach the parasite to the host root and provide the foundation for invasive cells internal to the haustorium to penetrate host epidermal and cortical cell layers (Baird and Riopel, 1984). Upon contact with the host stele, the haustorium develops a vascular bridge connecting host and parasite vascular systems.

Several phenols and quinones have been identified that induce haustorium development in vitro (Steffens et al., 1982; Chang and Lynn, 1986; Albrecht et al., 1999). Xenognosin activity is redox dependent, and phenols need to be oxidized to their sister quinones to be active haustorium inducers. In *Striga*, this reaction is driven by hydrogen peroxide-dependent peroxidases acting on monolignol molecules in the host cell walls (Smith et al., 1990). The correlation between quinone redox potential and inducing activity suggests that a key feature of xenognosins is their participation in oxidoreduction reactions (Smith et al., 1996). The involvement of radical semiquinones in haustorium signaling was suggested by the inhibitory effect of cyclopropyl-*p*-benzoquinone, a spin trap whose single electron reduction generates a reactive electrophile at the cyclopropyl ring that is predicted to irreversibly bind the xenognosin receptor (Zeng et al., 1996; Keyes et al., 2001). The requirement for a semiquinone was shown genetically by RNA interference (RNAi) silencing of quinone oxidoreductases in roots of the hemiparasite *Triphysaria versicolor* (Bandaranayake et al., 2010). Two xenognosin-regulated quinone oxidoreductases, one catalyzing single electron reductions and the other bivalent reductions, were silenced in transgenic *T. versicolor* roots. Silencing the single electron-reducing quinone oxidoreductase reduced haustorium development, while silencing the bivalent reducing enzyme had no effect. These results suggest that the first step in haustorium signaling is the redox activation of xenognosins to their semiquinone states (Bandaranayake et al., 2010).

We took a transcriptomic approach to identify parasite genes associated with xenognosin signaling (Matvienko et al., 2001a; Torres et al., 2005; Westwood et al., 2010). One interesting gene candidate for haustorium signaling is *TvPirin* (Matvienko et al., 2001a). Pirin was first described as a human protein that interacts with the transcription factor NF I in yeast two-hybrid systems (Wendler et al., 1997). In another two-hybrid screen, Pirin was identified as binding the oncoprotein Bcl3, a modulator of NF- $\kappa$ B transcription factor activity (Dechend et al., 1999). The Pirin protein is more abundant in C-Jun and rat sarcoma-transformed rat fibroblasts than in normal cells (Bergman et al., 1999). In *Serratia marcescens*, Pirin modifies pyruvate catabolism by interacting with pyruvate dehydrogenase (Soo et al., 2007). Pirins can also function catalytically as quercetin dioxygenases (Adams and Jia, 2005). The crystalline structure of human Pirin indicates two similar  $\beta$ -barrel domains arranged face to face with an iron cofactor within a cavity formed at the N-terminal do-

main, suggesting possible involvement in redox reactions (Pang et al., 2004).

The Arabidopsis (*Arabidopsis thaliana*) protein AtPirin1 binds the G $\alpha$ -subunit (GPA1) of the heterotrimeric guanine nucleotide binding protein (G protein) in yeast two-hybrid and in vitro binding assays (Lapik and Kaufman, 2003). The Pirin/GPA1 complex acts in concert with the NF-Y transcription factor to regulate the abscisic acid and blue light induction of the *light-harvesting chlorophyll a/b-binding* gene in Arabidopsis (Warpeha et al., 2007). Plants homozygous for *atpirin1* have delayed germination, inhibited seedling development, and earlier flowering times than the wild type (Lapik and Kaufman, 2003). The tomato (*Solanum lycopersicum*) pirin *Le-Pirin* is transcriptionally induced during camptothecin-induced programmed cell death (Orzaez et al., 2001). Because Pirin is transcriptionally regulated during haustorium development and is associated with signal transduction in plants, we investigated its function in parasitic plants.

## RESULTS

### *TvPirin* Gene Structure and Phylogeny

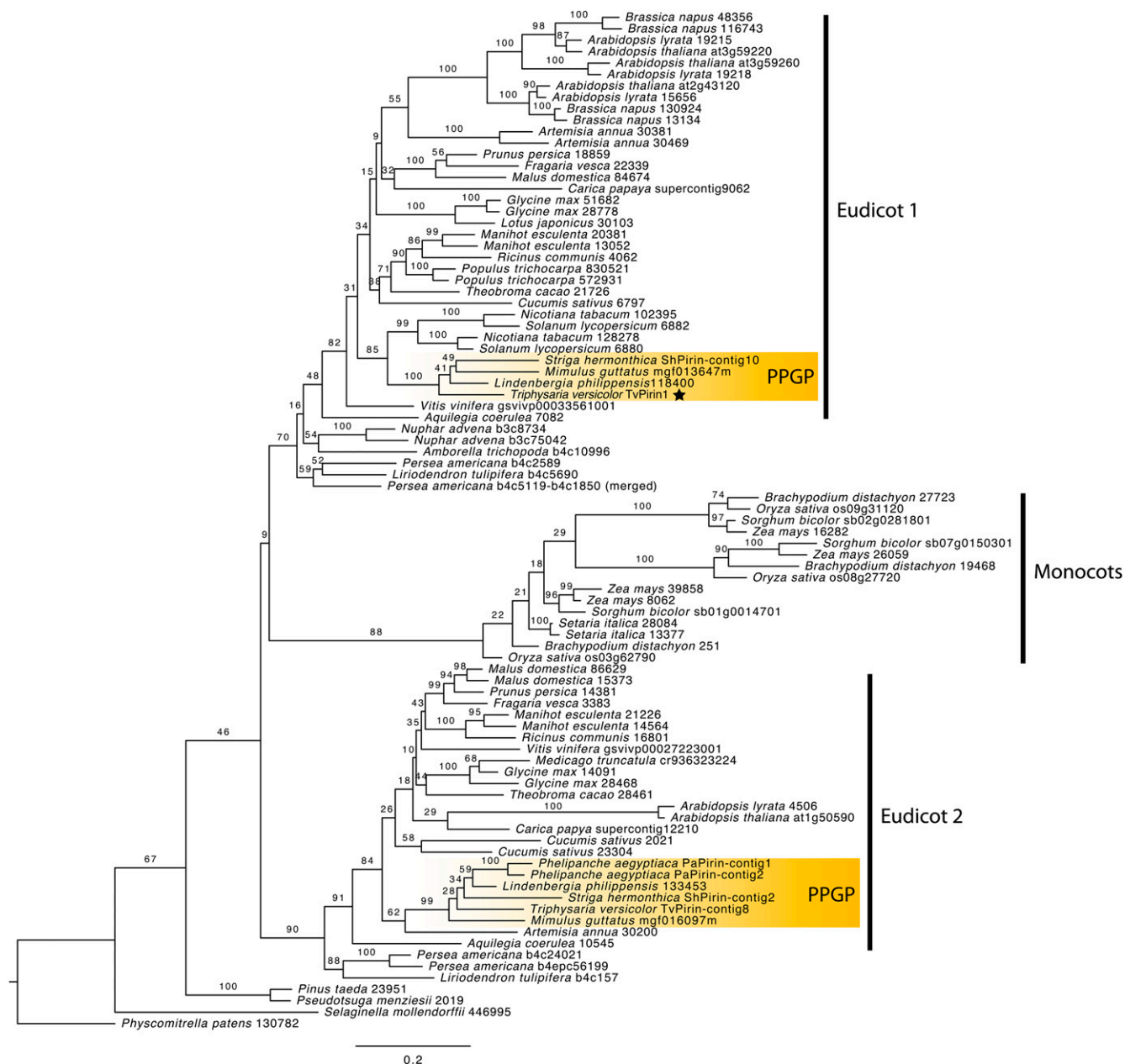
*TvPirin* was originally identified from a cDNA library enriched for transcripts up-regulated in *T. versicolor* root tips exposed to 2,6-dimethoxybenzoquinone (DMBQ; Matvienko et al., 2001a). The original *TvPirin* cDNA (BE574904.1) was predicted to be missing about 30 nucleotides of 5' coding sequences, so RACE reactions and sequence assemblies were carried out to obtain the complete coding sequence. The transcript is predicted to encode a 322-amino acid protein with about 70% amino acid identity to the human Pirin (NP\_003653.1; Wendler et al., 1997). There was no evidence of a nuclear localization signal using the protein localizations predictor WoLF PSORT (Horton et al., 2007).

Primers were designed from each end of the cDNA and used to amplify and sequence *T. versicolor* genomic DNA. Comparison of the genomic and cDNA sequences indicated that *TvPirin* has six introns. Two Arabidopsis *Pirin* homologs, At3g59260 and At2g43120, also have six introns located at analogous positions as in *TvPirin*. The Arabidopsis AtPirin1 characterized as a G $\alpha$  binding protein (At3G59220) has slightly less homology to *TvPirin* and has five introns at distinct positions from those in *TvPirin*. Therefore, it is unlikely that the characterized Arabidopsis gene *AtPirin1* is the ortholog of *TvPirin*.

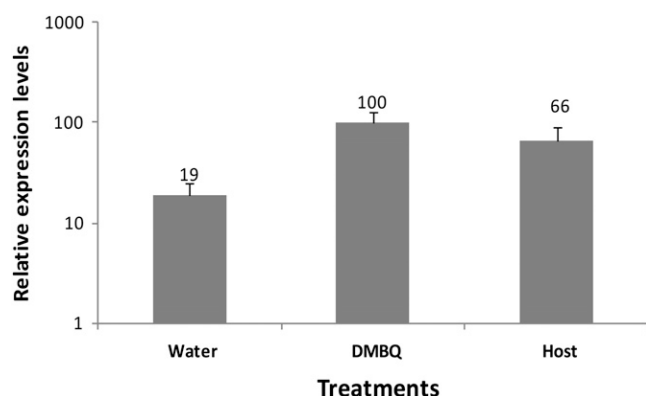
We searched the Parasitic Plant Genome Project (PPGP) EST database using BLASTN to determine how many homologs of *TvPirin* are present in Orobanchaceae. At the time of this analysis, the database reflected >1.3 billion reads derived from Sanger, 454, and Illumina cDNA sequencing of various tissues and growth stages of *T. versicolor*, *Striga hermonthica*, *Phelipanche aegyptiaca*, and *Lindenbergia philippensis* (Westwood

et al., 2010). We also searched for homologous sequences in public databases containing fully sequenced plant genomes to develop a phylogeny of plant *Pirins* (Fig. 1). This analysis indicated two clades of *Pirins* in eudicots, both represented in the Orobanchaceae. The *TvPirin* in this study (starred in Fig. 1) is part of a well-supported clade that includes the parasite *S. hermonthica* and the nonparasitic plants *Mimulus guttatus* and *L. philippensis*. Surprisingly, we did not detect a *P. aegyptiaca* homolog in this clade, but there was a *P. aegyptiaca*

homolog in clade 2. There is 77% amino acid identity between the clade 1 protein (TvPirin1) and clade 2 protein (TvPirin-contig 8). We cannot say if the clade 2 transcript is silenced by the hairpin constructions because the primers used in the quantitative reverse transcription (qRT)-PCR analyses were not designed to amplify TvPirin-contig8 transcripts. There was no evidence of parasite-specific duplications or expansions of *Pirin* as might be expected if it evolved novel functions in the parasite clade.



**Figure 1.** Pirin phylogeny. The phylogeny of the *Pirin* gene family was derived from sequence data of 10 fully sequenced plant genomes together with sequences from the PPGP (shaded and labeled PPGP). The *TvPirin* gene described in this manuscript is marked with a star. [See online article for color version of this figure.]



**Figure 2.** Transcriptional regulation of *TvPirin*. RNA was extracted from *T. versicolor* seedlings after exposure to water, DMBQ, or *M. truncatula* (host) roots for 2 h. Steady-state transcript levels of *TvPirin* were determined by qRT-PCR and normalized to the constitutively expressed gene *TvQN8* for each sample. Data are means ± SD of three technical replicates of three biological replicates ( $n = 9$ ). Each biological replicate was a square petri dish with 20 to 50 seedlings. Expression levels of roots treated with DMBQ was set to 100%. Note the relative expression values are written on top of each bar.

### Hairpin-Mediated Silencing of *TvPirin* Reduces Haustorium Development

Prior investigations showed that *TvPirin* is transcriptionally up-regulated in parasite roots by DMBQ (Matvienko et al., 2001a). In this study, we quantified the degree of *TvPirin* regulation in response to host root contact by qRT-PCR. As seen in Figure 2, contact with *Medicago truncatula* roots resulted in about a 3- to 4-fold increase in *TvPirin* transcripts in *T. versicolor* root tips, about the same as obtained with DMBQ.

A hairpin RNAi vector targeted against *TvPirin* sequences, called pHpPRN, and the parent vector, pHG8-YFP, were transformed into *T. versicolor* hypo-

cotyls via *Agrobacterium rhizogenes* and transgenic roots identified by their yellow fluorescent protein (YFP) fluorescence. Based on YFP fluorescence, 25% to 30% of the seedlings had at least one transgenic root. Roots transgenic for pHpPRN or pHG8-YFP had similar growth rates and morphologies (Table I).

RNA was harvested from pHG8-YFP and pHpPRN roots with and without DMBQ exposure and *TvPirin* transcripts quantified. Basal levels of *TvPirin* were about 20-fold lower in pHpPRN roots than pHG8-YFP roots (Fig. 3). Similar reductions were obtained in previous experiments targeting *TvQR1* and *TvQR2* by RNAi (Bandaranayake et al., 2010). When pHG8-YFP roots were exposed to DMBQ, there was a 3- to 4-fold induction of *TvPirin* compared to the water treatment. When pHpPRN roots were similarly treated with DMBQ, we detected no up-regulation of *TvPirin* (Fig. 3).

The ability of transgenic roots to develop haustoria was investigated by exposing transgenic roots to host roots, host root exudates, DMBQ, or water and assaying haustorium development 24 h later (Table II). Nontransgenic seedlings and control transgenic roots formed haustoria in approximately 70% to 85% of the roots after treatment with host root, root exudates, or DMBQ. In contrast, only about 20% to 50% of roots transformed with pHpPRN developed haustoria. No haustoria were seen to develop after the 24-h assay period.

### Hairpin-Mediated Silencing and Regulation of Additional Genes by *TvPirin*

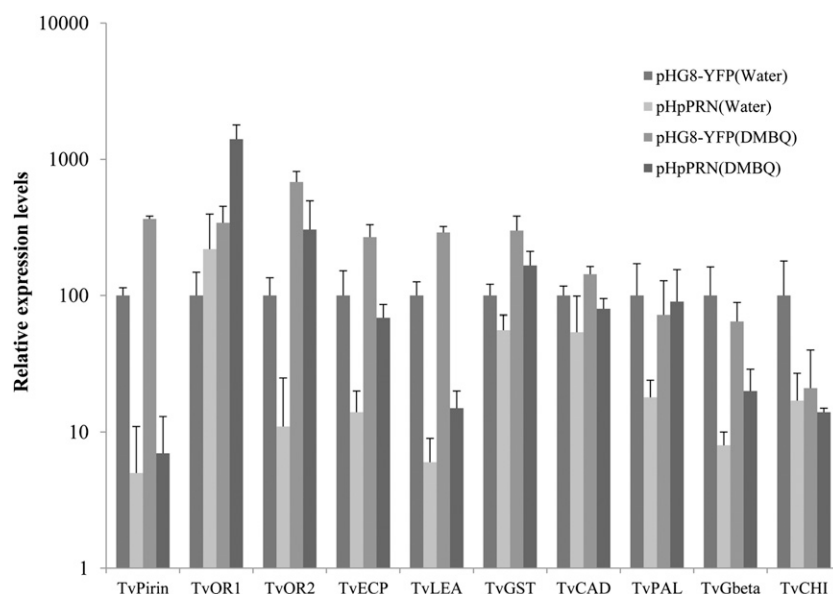
We determined steady state levels of 10 transcripts (including *TvPirin*) in *T. versicolor* roots transformed with either the empty parent vector (pHG8-YFP) or the hairpin construct (pHpPRN) by quantitative real-time PCR (Fig. 3). Five genes were up-regulated by DMBQ:

**Table I.** Efficiency and phenotypes of transgenic roots<sup>a</sup>

Phenotype	pHG8-YFP	pHpPRN	t-Value
Transformation efficiency <sup>b</sup>	28 ± 2.2 $n = 1271$	26 ± 2.8 $n = 1539$	0.61
YFP-positive roots per plant <sup>c</sup>	1.8 ± 0.4 $n = 352$	1.7 ± 0.4 $n = 403$	0.88
YFP-positive roots per plant <sup>d</sup>	2.0 ± 0.9 $n = 49$	2.4 ± 0.7 $n = 57$	0.05
Root length (cm) <sup>d</sup>	3.5 ± 1.8 $n = 49$	3.1 ± 1.0 $n = 57$	0.74
Lateral roots per YFP root <sup>d</sup>	0.1 ± 0.3 $n = 49$	0.1 ± 0.4 $n = 57$	0.20
Root growth no DMBQ (mm/24 h) <sup>d</sup>	2.6 ± 0.4 $n = 40$	2.5 ± 0.6 $n = 40$	0.85
Root growth with DMBQ (mm/24 h) <sup>d</sup>	2.7 ± 0.5 $n = 40$	2.6 ± 0.7 $n = 40$	0.12

<sup>a</sup>Values are mean ± SD with  $n$  = total number of plants or roots in all replicates. Means are the averages of three independent transformation experiments per construction. Data were analyzed using SAS statistical software (pooled  $t$  test/equal/unequal variance). <sup>b</sup>Percentage of plants with at least one yellow root. <sup>c</sup>Twenty-one days after transformation. <sup>d</sup>Thirty-three days after transformation.

**Figure 3.** Gene expression in pHpPRN roots. Steady-state transcript levels of 10 parasite genes in *TvPirin*-silenced roots were determined by quantitative PCR and normalized to the constitutively expressed gene *TvQN8* for each sample. Data are means  $\pm$  SD of three technical replicates of three to four plants ( $n = 9$ –12). Expression levels in pHG8-YFP transgenic roots treated with water was set to 100% and considered as the basal level of expression of the interested gene. Note the log scale y axis.



*TvPirin*, *TvQR1*, *TvQR2*, *TvGST*, *TvLEA*, and *TvECP*. The genes *TvQR1* and *TvQR2* encode quinone oxidoreductases and previously were reported as up-regulated by DMBQ (Matvienko et al., 2001b; Bandaranayake et al., 2010). *TvGST* encodes a putative glutathione transferase, a ubiquitous class of enzymes involved in detoxification by conjugation (Hayes et al., 2005). *TvLEA* is homologous to genes encoding Late Embryogenesis Abundant (LEA) proteins in Arabidopsis. Both glutathione *S*-transferase (*GST*) and *LEA* transcripts are differentially regulated in nonparasitic plants by various biotic and abiotic stresses (Mowla et al., 2006; Sappl et al., 2009). *TvECP* has weak homology to several putative plant cytochrome P450 monooxygenases. We also examined the expression of four genes not up-regulated by DMBQ: *TvCAD*, *TvPAL*, *TvCHI*, and *TvGbeta*. *TvPAL* is predicted to encode Phe ammonia-lyase and *TvCHI* chalcone isomerase, two genes on the phenylpropanoid biosynthesis pathway (Dixon and Paiva, 1995). *TvCAD* is a putative cinnamyl-alcohol dehydrogenase that catalyzes monolignol biosynthesis, a key step in lignin biosynthesis (Raes et al., 2003). *TvGbeta* is predicted to encode the  $\beta$ -subunit of the G protein complex (Assmann, 2002).

The basal expression level of each gene was taken as the transcript abundance in control pHG8-YFP roots treated with water. Seven genes, *TvPirin*, *TvQR2*, *TvECP*, *TvLEA*, *TvPAL*, *TvGbeta*, and *TvCHI*, were significantly reduced in pHpPRN compared to control roots (Fig. 3). Because there is no apparent homology between pHpPRN and the other transcripts (except *TvPirin*), the reduction in gene expression is likely a secondary effect of *TvPirin* silencing. Basal expression of *TvQR1*, *TvGST*, and *TvCAD* was not significantly affected in *TvPirin*-silenced roots.

The relative expression levels after DMBQ exposure were then determined (Fig. 3). All of the transcripts, except *TvPirin*, that were induced by DMBQ in control

roots were similarly induced in pHpPRN roots. These results suggest that *TvPirin* functions in establishing the basal transcript levels of a set of genes in parasite roots. However, *TvPirin* does not affect the responsiveness of genes to DMBQ.

## DISCUSSION

The chemical influence of some plants on the growth and development of others has been appreciated for centuries (Willis, 1985). While exploiting the potential

**Table II.** Haustorium development in transgenic roots silenced for *TvPIRIN*

Treatment <sup>a</sup>	Percentage of Roots with Haustoria		
	Seedlings <sup>b</sup>	pHG8YFP	pHpPRN
30 $\mu$ M DMBQ	75 $\pm$ 11 $n = 565$	71 $\pm$ 12* $n = 532$	38 $\pm$ 10** $n = 505$
Host root exudates	85 $\pm$ 3 $n = 214$	73 $\pm$ 9* $n = 66$	18 $\pm$ 5** $n = 27$
Host roots	87 $\pm$ 8 $n = 90$	84 $\pm$ 9* $n = 113$	47 $\pm$ 10** $n = 23$
Water	0 $\pm$ 0 $n = 186$	0 $\pm$ 0 $n = 104$	0 $\pm$ 0 $n = 97$

<sup>a</sup>*T. versicolor* roots were exposed to DMBQ or host exudates and assayed 24 h later or exposed to Arabidopsis roots and assayed after 5 d. Data are averages  $\pm$  SD of three to seven plates with four to six independently transformed plants in each plate. Experiment with DMBQ treatment was repeated three times with plants from three independent transformation experiments. Each plant had 1 to 30 roots depending on the age of the plant ( $n$  = total number of roots in all replicates). Each treatment was applied to similarly aged plants from the same transformation experiment. Pairwise comparisons of treatments within a row labeled with a different number of asterisks are significantly different at  $\alpha = 0.05$  (*t* test). <sup>b</sup>Seedling data were not included in statistical analysis because they were differently aged.

of allelopathy to improve crop performance is often discussed, little is known about the mechanisms by which plants detect and process chemical signals from other plants. Haustorium development in response to xenognosins provides a useful model for investigating chemical signaling between plant roots in general. Both xenognosin response and allelopathy are mediated by similar molecules: quinones and oxidized phenols. Depending on its concentration, DMBQ can be either a developmental stimulant that induces haustorium development or a plant toxin (Tomilov et al., 2006). Parasite quinone oxidoreductases use both xenognosins and allelopathic phytotoxins as biochemical substrates (Wrobel et al., 2002; Bandaranayake et al., 2010). Furthermore, both allelopathy and haustorium induction are dependent upon the redox state of the chemical agent. Juglone, the active allelopathic agent from black walnut trees, is synthesized in the inactive hydrojuglone state that is then activated to the toxic state upon exposure to oxygen (Lee and Campbell, 1969). As discussed previously, haustorium development is also dependent on the redox state of the inducer. Similarities between quinone associated allelopathy and haustorium initiation led to the hypothesis that the processes may share common molecular mechanisms (Tomilov et al., 2006).

Haustorium development is a multistep process that requires the coordinated expression of a number of genes and pathways. Most of the processes associated with haustorium development have almost certainly been derived from autotrophic plant processes. In this study, we found that the *TvPirin* gene associated with haustorium development has homologs in autotrophic plants that must provide functions unrelated to parasitism. Other genes associated with haustorium development similarly have nonparasitic functions in autotrophic plants. Expansins, nonenzymatic proteins that promote cell wall loosening, are used in both parasitic and nonparasitic plant processes (O'Malley and Lynn, 2000; Wrobel and Yoder, 2001).  $\zeta$  Crystallin quinone oxidoreductases catalyze similar biochemical reactions in many organisms but have specific functions in triggering haustorium development in parasites (Wrobel et al., 2002; Bandaranayake et al., 2010). *TvPirin* provides both parasite specific and nonparasitic functions without apparent gene duplication.

Our results are consistent with *TvPirin* functioning in transcription regulation because its inhibition results in several transcripts being down-regulated. The basal expression levels of seven of 10 genes investigated were significantly lower in pHpPRN than pHG8-YFP roots. These included both DMBQ-induced and -noninduced genes, so there was no apparent specificity for quinone-responsive transcripts. However, DMBQ up-regulated genes maintained the same level of responsiveness in *TvPirin*-silenced roots as in control transgenics, suggesting the role of *TvPirin* is restricted to establishing basal levels of gene expression rather than xenognosin responsiveness. We propose that *TvPirin* is a generalized transcription factor

associated protein that functions in the transcription of several different genes, some of which are needed for haustorium development.

## MATERIALS AND METHODS

### Chemicals, Plants, and Genes

Seeds of the outcrossing species *Triphysaria versicolor* were collected from an open pollinated population near Napa, CA. DMBQ was purchased from Pfaltz & Bauer, dissolved in water at 30  $\mu$ M, filter sterilized, and stored at 4°C until used. PCR primers were designed using Primer 3 software (Rozen and Skaletsky, 2000) and synthesized by Integrated DNA Technologies. Primer sequences are shown in Supplemental Table S1.

### Phylogenetic Analyses

The Pirin gene family was identified from PlantTribes 2.0 ([http://fgp.bio.psu.edu/tribedb/10\\_genomes/index.pl](http://fgp.bio.psu.edu/tribedb/10_genomes/index.pl)), an objective classification of plant proteins based on a cluster analysis of the inferred proteins of 10 fully sequenced plant genomes (Wall et al., 2008). Using the PlantTribes annotations, a single Tribe (roughly analogous to a gene family) was identified that contained four *Arabidopsis* (*Arabidopsis thaliana*) Pirin protein sequences. All embryophyte protein sequences that comprise the Pirin Tribe were extracted from PlantTribes and aligned using MAFFT v6.717b (Katoh et al., 2002; Katoh and Toh, 2008). The alignment was used to make a profile hidden Markov model with which publicly available EST sequences (<http://www.plantgdb.org/>) and gene models from genome sequences (<http://www.phytozome.net/>; Schnable et al., 2009; Schmutz et al., 2010; Velasco et al., 2010; Vogel et al., 2010; Shulaev et al., 2011) were searched using HMMER version 3.0 to identify putative homologs to the Pirin gene family genome sequences (<http://hmmer.janelia.org/>). All ESTs that were searched against the HMM were first translated with GeneWise from the Wise2 package (Birney et al., 2004) using the *Vitis vinifera* sequence as a reference. Additional parasite sequences were generated through the PPGP, a National Science Foundation-funded program aimed at obtaining deep transcriptome data sets from several species of Orobanchaceae (Westwood et al., 2010). Putative Pirin homologs from all PPGP assemblies (individual tissues and combo builds) were reassembled for each species using CAP3 (Huang and Madan, 1999) to identify a nonredundant set of transcripts (i.e. consolidate contigs that remained unassembled due to minor polymorphisms or sequencing errors or insufficient depth for automated assembly). Contigs and singletons from these assemblies as well as from all additional genome and transcriptome sequences were aligned using MAFFT, and a preliminary phylogenetic analysis was carried out with RAXML v7.0.4 (Stamatakis, 2006) to identify and remove any contaminating host sequences, as well as nearly identical, unassembled sequences (likely due to sequencing error or putative alleles). The nonredundant alignment was then realigned using SATé (Liu et al., 2009) and adjusted manually. The phylogenetic tree was produced with RAXML, with 1000 fast bootstrap replicates.

### Plasmid Constructions

Hairpin constructions were made using the Gateway-compatible vector pHellsgate 8 that contained a YFP reporter gene for selection in *T. versicolor* roots (Helliwell et al., 2002; Subramanian et al., 2006; Bandaranayake et al., 2010). A 462-nucleotide region of the *TvPirin* open reading frame was amplified using PCR primers flanked by attB recombination sites (Supplemental Table S1). The gel-purified PCR products were recombined into the Gateway donor vector pDONR211 following the manufacturer's protocol for BP recombination (Invitrogen). After the pDONR construct was confirmed by sequencing, Gateway LR recombination was performed with pHG8-YFP generating the hairpin vector pHpPRN. The vector was confirmed by restriction digestions.

### *T. versicolor* Root Transformation

*T. versicolor* roots were transformed using an *Agrobacterium rhizogenes*-based protocol as described (Bandaranayake et al., 2010). The plasmid constructions were transformed into *A. rhizogenes* MSU440 by electroporation (Sonti et al., 1995). Roots were excised from aseptic *T. versicolor* seedlings and



the cut ends inoculated with MSU440 bearing the appropriate constructions. Seedlings were maintained on agar plates at 25°C for about 3 weeks, at which time transgenic roots were identified by visualization of YFP fluorescence with a Zeiss Stemi SV11 dissecting microscope equipped with an YFP filter set. Transformation efficiencies were calculated as the number of plants with at least one yellow root.

## Haustorium Assay

Root exudates were collected from *Medicago truncatula* plants grown in Magenta boxes containing 20 mL of 0.5% agar. After 10 to 14 d, plants were removed and the agar was centrifuged at 20,000 rpm for 30 min. The supernatant was filter sterilized and stored at 4°C.

Haustorium development was assayed as previously described (Jamison and Yoder, 2001). Transgenic roots were maintained on agar plates at 25°C. Two milliliters of DMBQ (30  $\mu$ M) or host root exudates were added to the roots on each plate and haustoria assayed 24 h later. Alternatively, haustoria were induced by overlaying *T. versicolor* roots on the surface of an agar plate with Arabidopsis roots for 5 d. In all cases, the number of YFP positive roots that made haustoria was expressed as a fraction of the total number of YFP roots treated.

## Transcriptional Analyses

*T. versicolor* roots were exposed to *M. truncatula* roots or DMBQ for various times before being flash frozen in liquid nitrogen and RNA isolated using the TRIzol reagent (Invitrogen). RNA was treated with DNaseI and further purified using RNeasy Mini Spin columns (Qiagen). One microgram of RNA from each sample was converted to cDNA using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). The reverse transcription reactions were diluted 20-fold and 2  $\mu$ L used for SYBR green-based quantitative PCR assays using a LightCycler480 real-time PCR system (Roche) or ABI 7300 quantitative PCR system (Applied Biosystems). The cycle conditions were similar for both systems: 94°C for 3 min as initial denaturation followed by 35 cycles of 94°C for 15 s, 58°C for 30 s for primer annealing, and 72°C for 15 s. Melting curves of PCR products were obtained by gradually heating to 95°C, and only those producing a single melting peak were considered in the analysis. Target gene expression was measured relative to the constitutively expressed gene TvQN8. For LightCycler480 data, expression calculations used the standard curve method, taking into account the efficiencies of the PCR reactions, calculated by log-linear regression LightCycler480 analysis software (Roche). The data obtained with ABI 7300 system were analyzed with SDS Software using the  $\Delta\Delta$  Ct ratio ratio real-time PCR method (relative quantification), which determines the  $\delta$  Ct of the gene of interest and TvQN8 and subtracts this from the analogous measurement obtained from the internal control (the  $\delta - \delta$  Ct).

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *TvPirin*, JN606867; *TvQR1*, AF304461; *TvQR2*, AF304462; *TvECP*, DR173551; *TvGST*, DR173703; *TvLEA*, DR172703; and *TvCAD*, DR175870.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Table S1.** The sequences of the primers used in these experiments.

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